AD_____

Award Number: DAMD17-01-1-0403

TITLE: Characterization and Use of Temperature-Sensitive Mutations of BRCA1 for the Study of BRCA1 Function

PRINCIPAL INVESTIGATOR: Blase C. Billack, Ph.D.
Alvaro Monteiro, Ph.D.

CONTRACTING ORGANIZATION: Strang Cancer Prevention Center
New York, New York 10021

REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20030214 100

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual Summary (1 July 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Characterization and Use of Temperature-Sensitive Mutations of BRCA1 for the Study of BRCA1 Function

5. FUNDING NUMBERS
DAMD17-01-1-0403

6. AUTHOR(S)
Blase C. Billack, Ph.D.
Alvaro Monteiro, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Strang Cancer Prevention Center
New York, New York 10021

E-Mail: billacb@rockefeller.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Several physiological roles of the breast and ovarian cancer susceptibility gene (BRCA1) have been described which include control of cell cycle, activation of transcription and modulation of DNA damage response. The mechanism by which BRCA1 mediates these processes remains unclear. We have identified and characterized a naturally-occurring cancer-predisposing allele of BRCA1 with a missense mutation that displays temperature-sensitive activity. When the carboxy-terminus of BRCA1 carrying the mutation was fused to a GAL4 DNA-binding domain and expressed in human embryonic kidney cells, it was able to activate transcription of a GAL4-responsive reporter gene in a temperature-sensitive manner. At the permissive temperature, transcription activity of this mutant was approximately equal to wild-type, while at the non-permissive temperature, its activity was diminished but not abolished. Based on this observation, we designed a yeast-based screen for additional temperature-sensitive mutations of the C-terminus of BRCA1. One of the mutants identified in yeast, H1686Q, was able to activate transcription of a luciferase reporter gene in human cells in a temperature-dependent manner. Unlike the naturally-occuring mutation, the H1686Q was active only at the permissive temperature. Studies using full-length BRCA1 constructs carrying the naturally-occurring mutation indicate that the cellular distribution of this mutated protein was similar to wild-type at both permissive and non-permissive temperatures. We have generated several stable cell lines that express the full-length naturally-occurring cancer-predisposing mutant protein and are presently using these cells to analyze the role of BRCA1 in transcription activation, cell cycle control and DNA damage response.

14. SUBJECT TERMS
cancer biology, BRCA1, transcription activation, gene expression, DNA repair

15. NUMBER OF PAGES
38

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Introduction.....4

Body.....4

Key Research Accomplishments.....5

Reportable Outcomes.....9

Conclusions.....10

References.....10

Appendices.....12

INTRODUCTION

Mutations in the breast and ovarian cancer susceptibility gene *BRCA1* are observed in approximately 45% of families with a high incidence of breast cancer and 75% of families with multiple cases of breast and ovarian cancer. Current evidence indicates that BRCA1 is involved in several cellular processes including transcription activation, cell cycle regulation, DNA-damage repair and maintenance of genomic stability(Monteiro,2000; Venkitaraman, 2002). Alterations in BRCA1 may result in disruption of any or all of these processes and lead to cancer.

The C-terminus of the BRCA1 protein contains two BRCT domains in tandem referred to as the BRCT-N and BRCT-C. Interestingly, BRCT domains are globular structures that have been observed in other unrelated proteins important in DNA-damage repair and cell cycle control (Koonin et al., 1996; Bork et al., 1997 Callebaut and Mornon, 1997). The C-terminal region of BRCA1, when fused to a GAL4 DNA-binding domain (GAL4-DBD), has been shown to activate expression of a luciferase reporter gene driven by a GAL4 promoter element and, interestingly, cancer-predisposing mutations within the BRCT domains abolish this activity (Monteiro, 1996; Monteiro, 1997; Vallon-Christersson et al., 2001).

As described in our original proposal, we have identified a cancer-predisposing temperature-sensitive mutation in the C-terminal region of BRCA1. This mutation, R1699W, is a single amino acid substitution from arginine to tryptophan and is found within a stretch of nine amino acids that are 100% conserved across species. We have observed that in both yeast and 293T human embryonic kidney cells, this mutant can activate transcription of the reporter to levels observed for wild-type at the permissive temperature (30°C). At 37°C, the transcriptional activation of this mutant is much impaired relative to wild-type activity. From these data we proposed that this conditional mutation could be used as a tool to more clearly understand the biochemical mechanism(s) underlying BRCA1 activity.

BODY

During the past year we focused on two of the three specific aims described in our proposal and in the work statement:

Task 1. **Confirm that the full-length cDNA encoding the R1699W mutation is temperature-sensitive for physiologically relevant transcription activity in HCC1937 cells (months 0-2).**

- Develop mammalian expression constructs containing tagged, full-length wild-type and mutant BRCA1 cDNA. The selected mutants are R1699W, R1699Q, M1775R and Y1853X.
- Transiently transfect the expression constructs and measure transcription activity at permissive and non-permissive temperatures from the mdm2promoter-luciferase reporter construct.

Task 2. Establish and characterize stable cell lines (in HCC1937 cells) expressing the BRCA1 constructs created in Task 1 (months 2-26).

- Count the number of cells in growing cultures for each cell line at permissive and non-permissive temperatures to obtain growth curves.
- Perform western blots of lysates harvested above to assess protein expression levels.
- Perform immunohistochemistry on cultured cells to determine the intracellular localization of BRCA1 proteins.

KEY RESEARCH ACCOMPLISHMENTS

Progress on goals defined in Task 1.

A BRCA1 wild-type full-length construct (aa. 1-1863) was kindly provided by Ralph Scully (Harvard Medical School). In this construct, BRCA1 is contained in the pCDNA3 mammalian expression vector which has an N-terminal HA tag downstream of a CMV promoter and the β-globin intron. C-terminal fragments of BRCA1 containing either R1699W, R1699Q, M1775R, or Y1853X mutations were amplified by PCR using primers containing terminal *Xba I* restriction sites. To prevent methylation of the full-length wild-type BRCA1 *Xba I* restriction site, located in the C-terminus of the protein, this construct was transformed into and isolated from Dam^- bacteria. Mutant BRCA1 full-length constructs were then created by subcloning the C-terminal fragments in frame to the C-terminal region of wild-type BRCA1 excised by *Xba I* digest. Using this approach, full-length constructs (aa. 1-1863) of mutant BRCA1 R1699W, R1699Q, M1775R and Y1853X were obtained and confirmed by sequence analysis.

These constructs were then used to measure transcription activation at permissive (30°C) and non-permissive temperatures (37°C) from the mdm2 promoter-luciferase reporter construct. Other temperatures inbetween the nonpermissive and permissive temperatures were not examined in these assays, as we had previously found that transcription activation of the luciferase reporter gene by the C-terminus BRCA1 R1699W mutation fused to

the GAL4-DBD occurred maximally at 30°C, with a continous decrease in activity as the temperature was increased(data not shown).

Initial studies using transient transfection assays examined transcriptional activation in the full-length context using human 293T (BRCA1-proficient) or HCC1937 (BRCA1-deficient) cells co-transfected with wild-type or mutant full-length BRCA1 and the mdm2-luciferase reporter. Analysis of these data revealed no significant increase above baseline among the different constructs in transcriptional activation of the reporter at all times and temperatures tested after transfection up to and including 36 hours. We found that extended exposure to 30°C (> 30 hr) was not well-tolerated by the cells. It is possible that incubation times longer than 36 hours are necessary to observe effects on transcriptional activation and that the lack of differences may be the result of slight toxicity to the cells at the permissive temperature.

It has been well-documented that wild-type BRCA1 can potentiate the activation of p53-dependent and interferon γ-dependent gene transcription (Zhang et al., 1998; Ouchi et al., 2000). Furthermore, activation of the mdm2-luciferase reporter by wild-type full-length BRCA1 and p53 has been previously described (Ouchi et al., 1998). Thus, the lack of differences in transcription activation among the wild-type and mutant constructs of BRCA1 which we observed in the present study may suggest that previous reports rely on overexpression of the BRCA1 protein and expression of our full-length constructs is too low to observe effects on transcriptional activity. This may be why we do not observe enhanced transcriptional activation of the mdm2-luciferase by even the wild-type relative to empty vector alone. Earlier studies in our laboratory have demonstrated that expression of a full-length fusion construct of wild-type BRCA1:GAL4-DBD in human cells is significantly lower than expression of the wild-type C-terminus:GAL4-DBD (unpublished observations). Western blot analysis of wild-type and full-length constructs in 293T and HCC1937 cells will soon be performed to examine the relative expression levels of these constructs.

To summarize, we have completed all of the components of Task 1. Nevertheless, we are performing some additional experiments to address the possibilities that were raised after the completion of the proposed experiments.

Progress on goals defined in Task 2.

Several stable clonal cell lines obtained from HCC1937 cells transfected with full length constructs of R1699W and selected with G418 have been established. We screened over twenty

different clones in this manner and from these we obtained five clonal cell lines that express the mutated R1699W BRCA1 protein, as determined by western blotting (Table 1). Figure 1 is a representative western blot depicting expression of the R1699W protein in cell lines RW10.8M and RW10.8N. Hela nuclear extracts was used as a positive control for antibody binding. In western blot analysis we utilzed a mouse monoclonal antibody against the N-terminal of human BRCA1 (Ab-1, Oncogene Research Products, Cambridge, MA). The R1699W mutation does not affect binding of the primary antibody and can be used to detect expression of the mutated protein. We also generated a cell line of pooled clones designated as HCC/RW10.8(pooled) which also was found to express the mutated protein. It was noted that in at least two of these cell lines, RW10.8H and RW10.8(pooled), expression of the mutated BRCA1 was present only during log-phase growth and not when the cells had reached confluency. Although we did observe expression of R1699W BRCA1 in lysates from log-growth phase and confluent 10.8M and 10.8N cells, expression was found to be higher during log-growth (data not shown). These observations are in accord with a previous study which demonstrated that expression of BRCA1 is cell-cycle dependent (Jin et al., 1997), and suggest that there may be post-translational mechanisms which regulate the expression of BRCA1.

In order to obtain a cell line that could better tolerate prolonged exposure to 30°C, several stable clonal cell lines obtained from L56BR cells, a recently-characterized BRCA1-deficient cell line (Hegardt et al., 2002), transfected with full length constructs of R1699W and selected with G418 have been established. We screened 15 different clones in this manner and obtained one clonal cell line that expressed the mutated R1699W protein (Table 1). We also co-transfected mouse fibroblasts deficient in both p21 and Brca1 (a gift from T. Ludwig, Columbia University) with full-length constructs of R1699W and a puromycin resistance gene, selected with puromycin and obtained 18 clonal cells lines which were pooled and found to stably-express the mutated protein (Table 1). As with the HCC1937 cells, these cells also demonstrated diminished growth after extended time periods at 30°C (> 30 hr, not shown). Based on these observations we hypothesize that expression of the R1699W mutation at the permissive temperature may result in slowed growth compared to the parental BRCA1-deficient cells and are currently exploring this possibility.

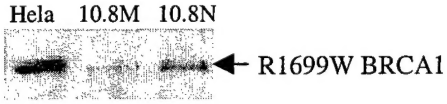
Table 1: Cell lines that stably-express R1699W BRCA1.

CLONE	Cell Type	LOG GROWTH	CONFLUENT
HCC/RW10.8B	Human Breast	+	ND
HCC/RW10.8J	Human Breast	+	ND
HCC/RW10.8H	Human Breast	+	-
HCC/RW10.8M	Human Breast	+	+
HCC/RW10.8N	Human Breast	+	+
HCC/RW10.8(pooled)	Human Breast	+	-
L56BR/RWjan4.E	Human Breast	+	+
MouseFibro/pooled	Mouse Fibroblast	+	ND

"+" indicates R1699W BRCA1 expression was detectable by western blotting. "-" indicates that expression of this protein was not observed. "ND" indicates that western blot analysis was not done.

Preliminary observations indicate that the cells which stably express the full-length R1699W mutation grow at similar rates as the parental HCC1937 cells at 37 C. At 30 C, both parental and stably-expressing cells grow significantly more slowly than at 37 C and both cell types appear to be dramatically affected by extended growth at 30 C. Immunohistochemical analysis for R1699W BRCA1 in HCC1937 transiently-transfected cells at both permissive and nonpermissive temperatures revealed that the protein is localized in a manner similar to wild-type and is predominately found in the nucleus (data not shown). This indicates that germ-line cancer-associated mutation of the R1699 residue does not affect cellular distribution of BRCA1 and suggests that the cancer-predisposing property of this mutation is not related to its cellular distribution.

Figure 1: Expression of R1699W BRCA1 in stable cell lines.



Cell lysates were prepared from log-phase cultures of RW10.8M and 10.8N and examined by western blotting. Nuclear extract obtained from Hela cells was used as a positive control for BRCA1 antibody binding.

At present, the other proposed stable cell lines of HCC1937 expressing full-length wild type BRCA1 and mutant M1775R and

Y1853X are being generated. Once all these cells are generated, western blotting will be performed to analyze relative expression of the wild-type and mutant full-length BRCA1 proteins. Immunohistochemistry experiments to assess cellular localization of these proteins will then be conducted.

To summarize, we have completed the components of Task 2 for the first year as outlined in the original proposal. The remaining experiments in this task are on schedule to be completed according to the outline proposed in the original grant application.

ADDITIONAL ACHIEVEMENTS

Based on our observation of a temperature-sensitive mutation in the C-terminus of BRCA1, we established a mutagenesis screen to identify more mutations of this region which could also activate transcription in a temperature-sensitive manner. We have idenified several mutations of this region which activate transcription of a reporter gene in yeast in a temperature-dependent fashion. One mutation in particular, H1686Q, was able to activate transcription of a reporter gene in both yeast and human embryonic kidney cells only at the permissive temperature (30°C). As with the R1699W, we postulate that the H1686Q mutation may act as a tool to dissect the biochemical role of BRCA1. Of particular interest is that unlike the C-terminal R1699W mutant, which exhibits some residual transcription activity at the nonpermissive temperature, the C-terminal H1686Q mutation displays no detectable activity at the nonpermissive temperature. A manuscript further characterizing the H1686Q mutation(Cancer Biology and Therapy, in press) is attached in the appendix. Based on our observations concerning the H1686Q mutant, we plan to clone the full length mutation and perform similar experiments as those outlined for the R1699W.

REPORTABLE OUTCOMES

- Full-length constructs of mutant BRCA1 R1699W, M1775R or Y1853X have been developed. Constructs are HA-tagged and contained within the pCDNA3 mammalian expression vector.

- We have established several cell lines of human HCC1937 which stably-express mutated full-length BRCA1 R1699W. We have also generated cell lines of human L56BR and mouse fibroblasts which express this mutant protein.

- Article entitled "Mutations in the BRCT Domain Confer Temperature Sensitivity to BRCA1 in Transcription

Activation." *Cancer Biology and Therapy*, in press. (DoD support is acknowledged; copy attached as an appendix).

SUMMARY OF TASKS

***Task 1.* Confirm that the full-length cDNA encoding the R1699W mutation is temperature-sensitive for physiologically relevant transcription activity in HCC1937 cells (months 0-2).**

We have completed all of the components of Task 1. *Nevertheless, we are performing some additional experiments to address the possibilities that were raised after the completion of the proposed experiments.*

Task 2. Establish and characterize stable cell lines (in HCC1937 cells) expressing the* BRCA1 *constructs created in Task 1 (months 2-26).

We have completed the components of Task 2 for the first year as outlined in the original proposal. The remaining experiments in this task are on schedule to be completed according to the outline proposed in the original grant application.

CONCLUSIONS

In the first year of our project, we were able to generate full-length constructs of a temperature-sensitive mutant and several other mutants of BRCA1. We have begun to use these constructs to study the transcriptional activating properties of this gene product in the full-length context. We are currently on schedule to complete all proposed studies as was originally outlined.

REFERENCES

Bork P, Hofmann K, Bucher P, Neuwald AF, Altschul SF, Koonin EV: **A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins**. *FASEB J* 1997, **11:**68-76.

Callebaut I, Mornon JP: **From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair**. *FEBS Lett.* 1997, **400:**25-30.

Hegardt C, Johannsson OT, Oredsson SM: **Rapid caspase-dependent cell death in cultured human breast cancer cells induced by the polyamine analogue N(1), N(11)-diethylnorspermine.** *Eur J Biochem* 2002, **269:** 1033-1039.

Jin Y, Xu XL, Yang MC, Wei F, Ayi TC, Bowcock AM, Baer R: **Cell cycle-dependent colocalization of BARD1 and BRCA1 proteins in discrete nuclear domains.** *Proc Natl Acad Sci U S A* 1997, **94**:12075-12080.

Monteiro AN, August A, Hanafusa H: **Common BRCA1 variants and transcriptional activation [letter].** *Am J Hum Genet* 1997, **61:**761-762.

Monteiro AN: **BRCA1: exploring the links to transcription.** *Trends Biochem.Sci* 2000, **25:**469-474.

Ouchi T, Monteiro AN, August A, Aaronson SA, Hanafusa H: **BRCA1 regulates p53-dependent gene expression.** *Proc Natl Acad Sci U S A* 1998, **95:**2302-2306.

Ouchi T, Lee SW, Ouchi M, Aaronson SA, Horvath CM: **Collaboration of signal transducer and activator of transcription 1 (STAT1) and BRCA1 in differential regulation of IFN-gamma target genes.** *Proc Natl Acad Sci U S A* 2000, **97:** 5208-5213.

Vallon-Christersson J, Cayanan C, Haraldsson K, Loman N, Bergthorsson JT, Brondum-Nielsen K, Gerdes AM, Moller P, Kristoffersson U, Olsson H, Borg A, Monteiro AN: **Functional analysis of BRCA1 C-terminal missense mutations identified in breast and ovarian cancer families.** *Hum Mol.Genet* 2001, **10:**353-360.

Venkitaraman, AR: **Cancer susceptibility and the functions of BRCA1 and BRCA2.** *Cell* 2002, **108**: 171-182.

Zhang H, Somasundaram K, Peng Y, Tian H, Zhang H, Bi D, Weber BL, El Deiry WS: **BRCA1 physically associates with p53 and stimulates its transcriptional activity.** *Oncogene* 1998, **16:**1713-1721.

Mutations in the BRCT Domain Confer Temperature Sensitivity to BRCA1 in Transcription Activation

Marcelo A. Carvalho[*1], Blase Billack[*], Emily Chan[*2], Terri Worley, Charmagne Cayanan and Alvaro N.A. Monteiro[†]

Strang Cancer Prevention Center, New York, NY 10021; Department of Cell and Developmental Biology, Weill Medical College of Cornell University, New York, NY 10021.

[*] These authors contributed equally to this work

Present address:
[1]Laboratório de Metabolismo Macromolecular Firmino Torres de Castro
Instituto de Biofísica Carlos Chagas Filho, UFRJ, Rio de Janeiro, Brazil

[2]Memorial Sloan Kettering Cancer Center
1275 York Avenue, New York, NY 10021

[†]Correspondence:
Alvaro N.A. Monteiro
Strang Cancer Research Laboratory
The Rockefeller University – Box 231
1230 York Ave., New York, NY 10021
Phone: 212- 7340567 ext. 225
Fax: 212-4729471
E-mail: monteia@rockvax.rockefeller.edu

Running title: Temperature-sensitive mutants of BRCA1

Carvalho, M.A., Billack, B., Chan, E., Worley, T., Cayanan, C. and Monteiro, A.N.A.

Mutations in the BRCT Domain Confer Temperature Sensitivity to BRCA1 in Transcription Activation

Cancer Biology and Therapy

ABSTRACT

BRCA1 **is a tumor suppressor gene and germ line mutations account for the majority of familial cases of breast and ovarian cancer. There is mounting evidence that BRCA1 functions in DNA repair and transcriptional regulation. A major hurdle to dissect the role of BRCA1 is the lack of molecular reagents to carry out biochemical and genetic experiments. Therefore, we used random mutagenesis of the C-terminus of BRCA1 (aa 1560-1863) to generate temperature-sensitive (TS) mutants in transcription activation. We obtained 11 TS mutants in transcription that localized primarily to the hydrophobic core of the BRCT-N domain of BRCA1. One of the mutants, H1686Q, also displayed temperature-dependent transcription activation in human cells. These conditional mutants represent valuable tools to assess the role of BRCA1 in transcription activation.**

Key words: BRCA1, yeast, mammalian two-hybrid system, transcription, temperature-sensitive mutants, CtIP, BRCT domain.

INTRODUCTION

Germ-line mutations in *BRCA1* confer high risk for breast and ovarian cancer.[1,2] The molecular function of BRCA1 is not yet known but there is increasing evidence that it is involved in DNA damage repair and gene transcription.[3,4] Several lines of evidence support a direct role for BRCA1 in transcription. When fused to a heterologous DNA binding domain (DBD) the C-terminus of BRCA1 activates transcription from a reporter gene and the introduction of cancer-associated mutations, but not benign polymorphisms, abolish activation.[5-7] In addition, BRCA1 interacts with the RNA polymerase II and with several complexes involved in chromatin remodeling.[8-11] Ectopic expression of BRCA1 results in the transcription of genes involved in cell cycle control and DNA damage repair.[12-17] Interestingly, BRCA1 also interacts with CsTF50 in a complex that regulates mRNA processing pointing to a pleiotropic role in transcription.[18]

Despite the absence of *BRCA1* homologs in its genome, yeast has been an important model system to study BRCA1 as well as the function of several mammalian transcription factors.[19] Yeast has been utilized to perform structure-function analysis of BRCA1 in transcription as well as to probe its mechanisms of activation based on the correlation with the clinical data.[5,7,20-22] In addition, overexpression of human BRCA1 in yeast generates a small colony phenotype that has been proposed as a method to classify uncharacterized mutations in BRCA1.[23,24] Thus, despite its limitations, yeast is a defined system to analyze BRCA1 function and is adequate for the rapid screening of large mutant libraries.

A major hurdle to define the function(s) of BRCA1 is the lack of molecular tools. Temperature-sensitive (TS) mutants would be particularly useful for this analysis.

Recently, we have identified a *BRCA1* allele in a family with familial ovarian cancer that displays a temperature-sensitive phenotype in mammalian cells (Worley et al. unpublished results and ref. 21). Therefore, we hypothesized that a differential screen in yeast based on random mutagenesis would allow us to isolate additional TS mutants. We followed the same procedure we had previously used to generate loss-of-function mutants in transcription activation and performed parallel screens at 30°C and 37°C.[20] We utilized this yeast-based system to identify and characterize 11 TS mutations and 15 loss-of-function (LF) mutants of BRCA1. One of the TS mutations identified in the yeast screen was found to exhibit a similar phenotype in human cells. These mutants will allow the study of BRCA1 function in yeast and provide a basis for the development of novel conditional mutants for mammalian cells.

METHODS

Yeast. *Saccharomyces cerevisiae* strain EGY48 [*MATα, ura3, trp1, his3, 6 lexA* operator-*LEU2*] was co-transformed with the LexA fusion vectors and reporter plasmid pSH18-34, which has *lacZ* under the control of 8 LexA operators.[25,26] The LexA DBD fusion of wild type human BRCA1 C-terminus (aa 1560-1863) and two germ-line mutants of BRCA1, Y1853X and M1775R were used as controls. Competent yeast cells were obtained using the yeast transformation system (Clontech).

Error-prone PCR mutagenesis and screening. A 30-cycle PCR reaction (94°C denaturation; 55°C annealing; 72°C extension) was performed using *Taq* polymerase, p385-BRCA1 plasmid as a template and oligonucleotide primers (S9503101, 5'-CGGAATTCGAGGGAACCCCTTACCTG-3'; S9503098, 5'-GCGGATCCGTAGTGG CTGTGGGGGAT-3'). PCR products were gel purified and co-transformed in an equimolar ratio with an *NcoI*-linearized wild-type pLex9 BRCA1 (aa 1560-1863) plasmid and pSH18-34. Transformants carrying the mutagenized cDNAs were plated at 37°C or 30°C on plates lacking tryptophan and uracil and containing 80 mg/L X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), 2% Galactose, 1% Raffinose, 1X BU salts (1L of 10X BU salts: 70g $Na_2HPO_4.7H_2O$, 30g NaH_2PO_4). The X-gal plates allowed direct visualization and were scored after 6 days. Clones were recovered from yeast and sequenced.

Mammalian cell reagents. A region comprising the BRCA1 coding region containing the TS mutation in pLex9 vector was excised with *Eco*R1 and *Bam*H1 and subcloned in pGBT9 in frame with GAL4 DBD. The fusion GAL4 DBD: BRCA1 was then cut with *Hind*III and *Bam*H1 and ligated into pCDNA3. We used the reporter pG5E1bLuc, which

contains a firefly luciferase gene under the control of five GAL4 binding sites[27] and transfections were normalized using a dual luciferase system (Promega). For the mammalian two-hybrid system the pCDNA3 GAL4 DBD: BRCA1 (aa 1560-1863) and the constructs carrying different TS mutations were used as bait to test interaction against CtIP. The construct containing CtIP (aa 45-897) fused to the herpesvirus VP16 transactivation domain (aa 411-456) was used as target and the VP16 vector was used as negative control (gift from Richard Baer, Columbia University).[28] Human 293T cells were cultured in DMEM supplemented with 5% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were carried out in quadruplicates using Fugene 6 (Roche, Indianapolis, IN) at 37°C for 12 hr. Cells were then incubated at 30°C or 37°C and harvested 16 hr later.

RESULTS

Screen for TS mutants of BRCA1 in transcription activation. We screened ~ 3 x 10^6 independent clones and recovered 1,302 putative LF mutants at 37°C (Figure 1A). These colonies were then plated on fresh plates and incubated at 37°C and 30°C for confirmation (Figure 1B). All plates contained yeast expressing wild-type cDNA to control for the different activity of β-galactosidase at both temperatures. Several clones turned out to display either a loss-of-function (white clones) or wild-type (blue clones) phenotype at both temperatures. Plasmids were recovered, retransformed into yeast and their activity confirmed. Clones that failed to display a reproducible activity were discarded. Plasmids representing 38 clones (3 were not recovered) were analyzed by restriction digest and although no clone had detectable deletions/insertions by gel analysis, sequencing revealed that 12 had nucleotide deletions or nonsense mutations and were not analyzed further. The remaining clones were processed for sequencing and the mutation identified. Eleven clones displayed markedly reduced activity at 37°C and wild-type activity at 30°C (TS clones; Table 1) and 15 had reduced activity at both temperatures (LF clones, Table 2).

TS mutants in yeast. Our screen resulted in the isolation of 11 TS mutants (8 unique) in transcription activation in yeast (Table 1). Seven clones displayed only one missense mutation and four clones displayed two missense mutations (Table 1). It is unclear whether the two mutations are required for the TS phenotype or not. At least in one case, TS32 (S1722F/K1667E), we know this is not the case because a similar mutation was found independently in another clone, TS25. Mutations causing TS activity were found in

exons 16-20 and 24. Interestingly, conserved hydrophobic residues were found to be a major target of mutations followed by mutations in serine residues (Table 1 and Fig. 2). With three exceptions, S1631N, L1639S and E1836G, all mutations occurred either in the N-terminal BRCT region or in the interval between the N- and C-terminal BRCTs (Table 1 and Fig. 2).

Loss-of-function (LF) mutants. Due to the experimental design, several clones proved not to be TS mutants but instead LF mutants at both temperatures tested (Table 2). These mutations also targeted hydrophobic residues in the BRCT domains. Interestingly, we recovered a recurring cancer-associated mutation of BRCA1, M1775R (LF35; Table 2).[29] Also, Q1811R and A1843P, found together in LF2, are unclassified variants listed in the Breast Cancer Information Core database (http://www.nhgri.nih.gov/Intramural_research /Lab_transfer/Bic/). Two mutations, F1704S and L1657P, were found in 4 and 2 independent clones, respectively. Mutations causing LF phenotype were found in all exons examined with the exception of exon 19.

TS mutants in human cells. All unique TS clones had their activity measured in human cells using a fusion to GAL4 DBD and a luciferase reporter driven by a GAL4-responsive promoter. Negative controls used were two cancer-associated mutants, M1775R and Y1853X.[29,30] In four independent experiments, one of the mutants (TS50) reproducibly displayed significant activity at the permissive temperature. Whereas at 30°C it exhibited approximately 30% of wild-type activity, at 37°C it did not activate transcription of the reporter (Fig. 3). Western blot analysis revealed that all mutant constructs were being

expressed, albeit at lower levels than the WT protein (not shown).

Mammalian two-hybrid system. Recent reports have demonstrated that CtIP, a protein involved in transcriptional repression and a substrate of ATM, interacts with the BRCT domains of BRCA1.[28,31-34] We next examined whether the TS mutants could interact with CtIP in a temperature-dependent manner in a mammalian two-hybrid assay. We reasoned that this assay would provide a complementary approach to assess the temperature-sensitive phenotype of the mutants. Our results confirm previous reports that CtIP interacts with the carboxy-terminal region of BRCA1 and show that this interaction also occurs at 30°C [31](Fig. 4).[28] Interestingly, TS26 and TS50 were found to interact with CtIP only at 30°C. The fold induction relative to the activity of the TS mutants transfected with the VP16 transactivation domain alone (7 fold and 10 fold, respectively) was less than fold induction obtained with the WT and CtIP:VP16, suggesting that the interaction at 30°C is only partially restored. Although we observed that TS26 interacts with CtIP, it failed to activate transcription at either temperature (Fig. 3).

DISCUSSION

The function of BRCA1 has remained elusive despite extensive effort to characterize its biochemical activities. It has been implicated in DNA repair, transcription activation and repression, transcription-coupled repair, mRNA processing, cell cycle checkpoint regulation and ubiquitination.[3,4,18,35,36] We reasoned that the isolation of conditional mutants would be an important addition in the experimental armamentarium to study BRCA1. Here we developed a screening strategy to isolate mutants of the BRCA1 C-terminus that display a TS phenotype.

Our screen isolated 11 unique loss-of-function (LF) mutants (Table 2), extending our analysis of mutants that affect transcription activation by BRCA1 and allowing us to have a more detailed picture of the structure-function features of the C-terminal region of BRCA1.[20,21] The LF mutants recovered were localized primarily in conserved hydrophobic residues at the BRCT-N and the BRCT-C domains.

We have also isolated 8 unique TS mutants using the yeast screening (Table 1, Figure 2). One mutation, F1734L, was found in 4 independent clones in our TS set and two mutations, F1704S and L1657P, were found in 4 and 2 independent clones, respectively, in the loss-of-function set. These findings suggest that the screen might have reached saturation and therefore the mutants recovered identify important regions for the regulation of BRCA1. To understand the functional consequences of these mutations we mapped the mutations onto the crystal structure of the BRCT domain region of BRCA1 (Figure 2A).[37] Two mutations (S1631N and L1639S) mapped to regions outside the BRCT domain and were excluded from our analysis. Significantly, all other mutations leading to temperature sensitivity, with one exception (E1836G) mapped to secondary

structures in the BRCT-N and to the interval region (Figure 2B) and cluster preferentially at the hydrophobic core of the domain (Figure 2A). The reason for this clustering is not known but it is possible that mutations the BRCT-C have more dramatic consequences for the general folding and therefore are not stable even at lower temperatures. Alternatively, the BRCT-N may provide an important binding site to the RNA polymerase II holoenzyme, an idea that is corroborated by in vitro binding studies of BRCA1 and RNA helicase A.[9] Therefore, for mutations in the BRCT-C to affect transcription their effect has to be more dramatic allowing us to isolate only loss of function mutations.

Interestingly, mutations in residues located at hydrophobic cores in the catalytic domain of tyrosine kinases as well as in SH3 domains have been demonstrated to confer temperature-sensitivity.[38] In three clones (TS1, TS4 and TS36) two mutations were found and only one of them may be important for temperature sensitivity. Alternatively, as found in TS mutants of v-Src, two mutations may be required.[39,40]

One of the TS mutants isolated in yeast, H1686Q, displayed a temperature-dependent activation of transcription when tested in human cells (Fig. 3). This observation indicates that residue H1686 is located at a critical position for the stability of the BRCT domains (Fig.2). In addition to the ability of TS50 to activate transcription only at the permissive temperature in mammalian cells, we found that its interaction with CtIP also occurred in a temperature-dependent manner (Fig. 4). Intriguingly, mutant TS26 interacts with CtIP at the permissive temperature but is unable to activate transcription at either 30°C or 37°C. Based on these observations we propose that TS50 can be used to clarify the physiological relevance of the BRCA1/CtIP interaction.

The inability of most of these clones to behave as TS mutants in mammalian cells may be due to inherent differences in the range of temperatures and metabolism of yeast versus the mammalian system. Alternatively, this may reflect the fact that the reporter used in the screen is not stringent. We tend to favor the latter explanation because there are documented examples of TS mutants isolated in yeast screens at 25°C and 33°C, permissive and restrictive temperature respectively, that turned out to display TS activity in mammalian cells at 34°C and 40.5°C.[38] This is a striking example in which the permissive temperature in mammalian cells was even higher than the restrictive temperature in yeast suggesting that the mutants adapt to the range of temperatures used in a particular host. The use of a low-stringency reporter is important at the restrictive temperature to guarantee the selection of mutants with the lowest possible activity. However, when screened at the permissive temperatures it will allow the selection of clones that may have low activity. We are currently exploring these different possibilities.

Although only one of the mutant clones displayed a mammalian TS phenotype in transcription, the other clones isolated here are candidates to become molecular biological tools in yeast to dissect the function of BRCA1 in transcription and to guide further efforts to isolate more relevant TS mutants in mammalian cells. If we apply a conservative interpretation of the transcriptional assay, *i.e.* that it is a measure of the integrity of the BRCT domain, then it is possible that the data collected here may serve as a basis to rationally design conditional mutants to other proteins that present BRCT domains in their structure. It is important to stress that the TS mutants recovered are inactive at 37°C and are likely to represent cancer-associated variants if found as germ-line mutations.

ACKNOWLEDGEMENTS

Thanks to Claudia Bernardi and José Galán for critically reading the manuscript, Nebojsa Mirkovic for the preparation of figure 2A and Richard Baer for providing the CtIP constructs. This work was carried out with support from NIH 1RO1 CA92309 (A.N.A.M.), U.S. Army awards DAMD17-99-1-9389 (A.N.A.M.) and DAMD17-01-1-0403 (B.B.), a CAPES fellowship (M.A.C.) and the Fashion Footwear Association of New York/QVC.

REFERENCES

1. Easton DF, Bishop DT, Ford D, Crockford GP. Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. *Am J Hum Genet* 1993; 52:678-701.

2. Struewing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M et al. The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. *N Engl J Med* 1997; 336:1401-1408.

3. Monteiro AN. BRCA1: exploring the links to transcription. *Trends Biochem Sci* 2000; 25:469-474.

4. Venkitaraman AR. Cancer Susceptibility and the Functions of BRCA1 and BRCA2. *Cell* 2002; 108:171-182.

5. Monteiro AN, August A, Hanafusa H. Evidence for a transcriptional activation function of BRCA1 C-terminal region. *Proc Natl Acad Sci USA* 1996; 93:13595-13599.

6. Chapman MS, Verma IM. Transcriptional activation by BRCA1. *Nature* 1996; 382:678-679.

7. Monteiro AN, August A, Hanafusa H. Common BRCA1 variants and transcriptional activation. *Am J Hum Genet* 1997; 61:761-762.

8. Scully R, Anderson SF, Chao, DM, Wei W, Ye L, Young RA, et al. BRCA1 is a component of the RNA polymerase II holoenzyme. *Proc Natl Acad Sci USA* 1997; 94:5605-5610.

9. Anderson SF, Schlegel BP, Nakajima T, Wolpin ES, Parvin JD. BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A. *Nat Genet* 1998; 19:254-256.

10. Bochar DA, Wang L, Beniya H, Kinev A, Xue Y, Lane WS et al. BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. *Cell* 2000; 102:257-265.

11. Pao GM, Janknecht R, Ruffner H, Hunter T, Verma IM. CBP/p300 interact with and function as transcriptional coactivators of BRCA1. *Proc Natl Acad Sci USA* 2000; 97:1020-1025.

12. Somasundaram K, Zhang H, Zeng YX, HouvrasY, Peng Y, Zhang H et al. Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. *Nature* 1997; 389:187-190.

13. Ouchi T, Monteiro AN, August A, Aaronson SA, Hanafusa H. BRCA1 regulates p53-dependent gene expression. *Proc Natl Acad Sci USA* 1998; 95:2302-2306.

14. Harkin DP, Bean JM, Miklos D, Song YH, Truong VB, Englert C et al. Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. *Cell* 1999; 97:575-586.

15. MacLachlan TK, Somasundaram K, Sgagias M , Shifman Y, Muschel RJ, Cowan, KH et al. BRCA1 effects on the cell cycle and the DNA damage response are linked to altered gene expression. *J Biol Chem* 2000; 275:2777-2785.

16. Yarden RI, Pardo-Reoyo S, Sgagias M, Cowan KH, Brody LC. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. *Nat Genet* 2002; 30:285-289.

17. Takimoto R, MacLachlan TK, Dicker DT, Niitsu Y, Mori T, El Deiry WS. BRCA1 transcriptionally regulates damaged DNA binding protein (DDB2) in the DNA repair response following UV-irradiation. *Cancer Biology and Therapy* 2002; 1:177-186.

18. Kleiman FE, Manley JL Functional interaction of BRCA1-associated BARD1 with polyadenylation factor CstF-50. *Science* 1999; 285:1576-1579.

19. Kennedy BK. Mammalian transcription factors in yeast: strangers in a familiar land. *Nat Rev Mol Cell Biol* 2002; 3:41-49.

20. Hayes F, Cayanan C, Barilla D, Monteiro AN. Functional assay for BRCA1: mutagenesis of the COOH-terminal region reveals critical residues for transcription activation. *Cancer Res* 2000; 60:2411-2418.

21. Vallon-Christersson J, Cayanan C, Haraldsson K, Loman N, Bergthorsson JT, Brondum-Nielsen K et al. Functional analysis of BRCA1 C-terminal missense mutations identified in breast and ovarian cancer families. *Hum Mol Genet* 2001; 10:353-360.

22. Nadeau G, Boufaied N, Moisan A, Lemieux KM, Cayanan C, Monteiro AN et al. BRCA1 can stimulate gene transcription by a unique mechanism. *EMBO Reports* 20001:260-265.

23. Monteiro AN, Humphrey JS. Yeast-based assays for detection and characterization of mutations in BRCA1. *Breast Disease* 1998; 10:61-70.

24. Humphrey JS, Salim A, Erdos MR, Collins FS, Brody LC, Klausner RD. Human BRCA1 inhibits growth in yeast: potential use in diagnostic testing. *Proc Natl Acad Sci USA* 1997; 94:5820-5825.

25. Golemis EA, Gyuris J, Brent R. Two-hybrid system/interaction traps. In Ausubel FM, Brent R, Kingston R, Moore D, Seidman J, Smith JA, Struhl K (ed.), *Current Protocols in Molecular Biology.* John Wiley & Sons, New York. 1994

26. Estojak J, Brent R, Golemis EA. Correlation of two-hybrid affinity data with in vitro measurements. *Mol Cell Biol* 1995; 15:5820-5829.

27. Seth A, Gonzalez FA, Gupta S, Raden DL, Davis RJ. Signal transduction within the nucleus by mitogen-activated protein kinase. *J Biol Chem* 1992; 267:24796-24804.

28. Yu X, Wu LC, Bowcock AM, Aronheim A, Baer R. The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. *J Biol Chem* 1998; 273:25388-25392.

29. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994; 266:66-71.

30. Friedman LS, Ostermeyer EA, Szabo CI, Dowd P, Lynch ED, Rowell SE et al. Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nat Genet* 1994; 8:399-404.

31. Wong AK, Ormonde PA, Pero R, Chen Y, Lian L, Salada G et al. Characterization of a carboxy-terminal BRCA1 interacting protein. *Oncogene* 1998; 17:2279-2285.

32. Li S, Chen PL, Subramanian T, Chinnadurai G, Tomlinson G, Osborne CK et al. Binding of CtIP to the BRCT repeats of BRCA1 involved in the transcription regulation of p21 is disrupted upon DNA damage. *J Biol Chem* 1999; 274:11334-11338.

33. Li S, Ting NS, Zheng L, Chen PL, Ziv Y, Shiloh Y et al. Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response. *Nature* 2000; 406:210-215.

34. Wu-Baer F, Baer R. Tumour suppressors (Communication arising): Effect of DNA damage on a BRCA1 complex. *Nature* 2001; 414:36.

35. Baer R, Ludwig T. The BRCA1/BARD1 heterodimer, a tumor suppressor complex with ubiquitin E3 ligase activity. *Curr Opin Genet Dev* 2002; 12:86-91.

36. Aprelikova ON, Fang BS, Meissner EG, Cotter S, Campbell M, Kuthiala A et al. BRCA1-associated growth arrest is RB-dependent. *Proc Natl Acad Sci USA* 1999; 96:11866-11871.

37. Williams RS, Green R, Glover JNM. Crystal structure of the BRCT repeat region from the breast cancer-associated protein BRCA1. *Nat Struct Biol* 2001; 8:832-842.

38. Parrini MC, Mayer BJ. Engineering temperature-sensitive SH3 domains. *Chem Biol* 1999; 6:679-687.

39. Mayer BJ, Jove R, Krane JF, Poirier F, Calothy G, Hanafusa H. Genetic lesions involved in temperature sensitivity of the src gene products of four Rous sarcoma virus mutants. *J Virol* 1986; 60:858-867.

40. Nishizawa M, Mayer BJ, Takeya T, Yamamoto T, Toyoshima K, Hanafusa H et al. Two independent mutations are required for temperature-sensitive cell

transformation by a Rous sarcoma virus temperature-sensitive mutant. *J Virol* 1985; 56:743-749.

Table 1. Temperature-sensitive mutants of BRCA1 (aa 1560-1863) in transcription.

Clone	Exon	Mutation	Nucleotide change[a]	Allowed residues[b]	Secondary structure[c] and comments	Activity[d] 30°C	37°C
TS1	17	F1668S	T5122C	F	BRCT-N α-helix 1	+++	-
	24	E1836G	A5626G	DE	BRCT-C α-helix 3		
TS4	16	L1605L	T4932C	silent	unknown	+++	-
	17	V1687A	T5179C	V	BRCT-N β-sheet 3		
	19	K1727E	A5298G	KRQ	BRCT-N/BRCT-C interval		
TS6	16	L1639S	T5035C	LV	unknown	+++	-
TS19	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
	20	E1735E	A5324G	silent	BRCT-N/BRCT-C interval		
TS25	16	S1610S	T4949C	silent	unknown	+++	-
	19	S1722F	C5284T	S	BRCT-N α-helix 3		
TS26	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS30	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS32	17	K1667E	A5118G	KR	BRCT-N α-helix 1	+++	-
	19	S1722F	C5284T	S	BRCT-N α-helix 3		
TS33	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS36	16	S1631N	G5011A	SI	unknown	+++	-
	18	V1713A	T5257C	VI	BRCT-N β-sheet 4; uncharacterized variant found as a germline mutation[e]		
TS50	17	H1686Q	T5177A	H	BRCT-N β-sheet 3	+	-

[a] Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession #U14680. [b]Residues that are found in the same position in an alignment of human (U14680), chimpanzee (AF207822), dog (U50709), rat (AF036760), mouse (U68174), chicken (AF355273) and frog (AF416868) homologs. [c]According to the BRCA1 BRCT crystal structure[37]. [d]Activity was scored in plates after 6 days. (+++) activity comparable to wild-type BRCA1. [e] As described in the Breast Cancer Information Core (BIC) database.

Table 2. Loss-of-function mutants of BRCA1 (aa 1560-1863) in transcription.

Clone	Exon	Mutation	Nucleotide change[a]	Allowed residues[b]	Secondary structure[c] and comments[e]	Activity[d] 30°C	37°C
LF2	23	Q1811R	A5551G	Q	BRCT-C β3-α2 loop; uncharacterized variant found as a germline mutation[e]	-	-
	23	P1812S	C5553T	P	BRCT-C β3-α2 loop		
	24	A1843P	G5646C	AS	BRCT-C α-helix 3; uncharacterized variant found as a germline mutation[e]		
LF3	17	L1671L	A5132G	silent			
	18	E1694G	A5201G	E	BRCT-N β3-α2 loop	-	-
	24	V1842A	T5644C	VIL	BRCT-C α-helix 3		
LF5	16	L1657P	T5089C	L	BRCT-N β1-α1 loop;	-	-
LF8	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF15	24	A1843T	G5646A	AS	BRCT-C α-helix 3	-	-
	17	T1691T	A5192G	silent	unknown		
LF20	17	F1668S	T5122C	F	BRCT-N α-helix 3		
	24	R1835R	A5624G	silent		-	-
	24	P1856T	C5685A	PQS	unknown		
LF22	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF23	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF24	20	G1743R	G5346A	G	BRCT-N/BRCT-C interval	-	-
	16	L1636L	T5034C	silent			
LF27	16	L1657P	T5089C	L	BRCT-N β1-α1 loop	-	-
	17	L1664L	C5111T	silent			
LF28	16	S1577P	T4848C	S	unknown		
	16	S1655P	T5082C	S	BRCT-N β1-α1 loop; residue mutated in the germline (S1655F)[e]	-	-
LF35	21	M1775R	T5443A	M	BRCT-C β1-α1 loop; cancer-associated mutation found in the germline[e]	-	-
	22	Q1779Q	A5456G	silent			
LF34	23	I1807S	T5539G	IVL	BRCT-C β-sheet 3		
	23	H1822H	T5585C	silent		-	-
LF38	16	E1660G	G5098A	EKSC	BRCT-N α-helix 1	-	-
LF47	16	R1649R	A5066G	silent			
	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-

[a] Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession #U14680. [b] Residues that are found in the same position in an alignment of human (U14680), chimpanzee (AF207822), dog (U50709), rat (AF036760), mouse (U68174), chicken (AF355273) and frog (AF416868) homologs. [c] According to the BRCA1 BRCT crystal structure[37]. [d] Activity

LEGENDS

Figure 1. Screening for temperature-sensitive mutants of BRCA1 in transcription. **A**. Primary screening at 37°C. Transformants carrying BRCA1 with wild-type activity appear as blue colonies (blue arrow) and transformants carrying loss-of-function mutants at 37°C appear as white colonies (white arrow). White colonies were replated in parallel and incubated at 30°C and 37°C. **B.** Plates containing replicas of each white clone isolated from primary plates. A transformant carrying a wild-type BRCA1 is included at the top of each plate (white arrow). Clones that were consistently white at 37°C and blue at 30°C were isolated as temperature-sensitive mutants (blue arrow). Clones that were white at both temperatures were isolated as loss-of-function mutants.

Figure 2. TS mutations localize primarily to the BRCT-N domain. A. The location of the eight unique TS mutations is shown in the BRCT-N and BRCT-C domains of human BRCA1 according to the crystal structure of human BRCA1 BRCT region.[37] Red spheres represent the only coding change in a single clone and blue spheres represent changes that are in clones with multiple mutations. Note that with exception of S1631N, L1639S (which precede the BRCT domains and are not shown) and E1836G (TS1), all other TS mutations map to the BRCT-N domain. **B.** Secondary structure elements according to crystal structure of BRCA1 BRCT region[37] are depicted above the sequence. Interval region, separating BRCT-N and BRCT-C is represented by a dotted line with a α-helix (αL; purple). Residue positions mutated in TS clones are shown for clones containing one (red triangle) or two changes (blue triangle). Changes are indicated below the sequence.

Figure 3. Transcriptional activity of TS mutants in mammalian cells. Upper panel shows a schematic representation of the GAL4-DBD:BRCA1 (aa 1560-1863) fusion protein and of the luciferase reporter gene driven by five GAL4 binding sites. Lower panel depicts the activation of luciferase expression by wild type and mutant BRCA1 constructs in 293T cells at 37° C (solid bars) or 30° C (open bars). Data were normalized to the percentage of wild-type activity at each temperature. MR, BRCA1 (aa 1560-1863) carrying the cancer-associated M1775R mutation used as negative control.

Figure 4. Mammalian two-hybrid system reveals a temperature-dependent interaction between BRCA1 TS mutants and CtIP. The upper panel shows a schematic representation of the GAL4-DBD:BRCA1 (aa 1560-1863) fusion protein used as bait, the CtIP:VP16 fusion protein used as target and the luciferase reporter gene driven by five GAL4 binding sites. The lower panel depicts the activation of the reporter gene at 37° C or 30° C by wild type and mutant BRCA1 constructs in 293T cells co-transfected with empty VP16 vector (that codes for the VP16 transactivation domain alone) or vector containing CtIP (that codes for the CtIP:VP16 fusion protein). The data were normalized to show the fold induction of transcriptional activity for each TS mutant relative to its activity when transfected with VP16 vector alone.

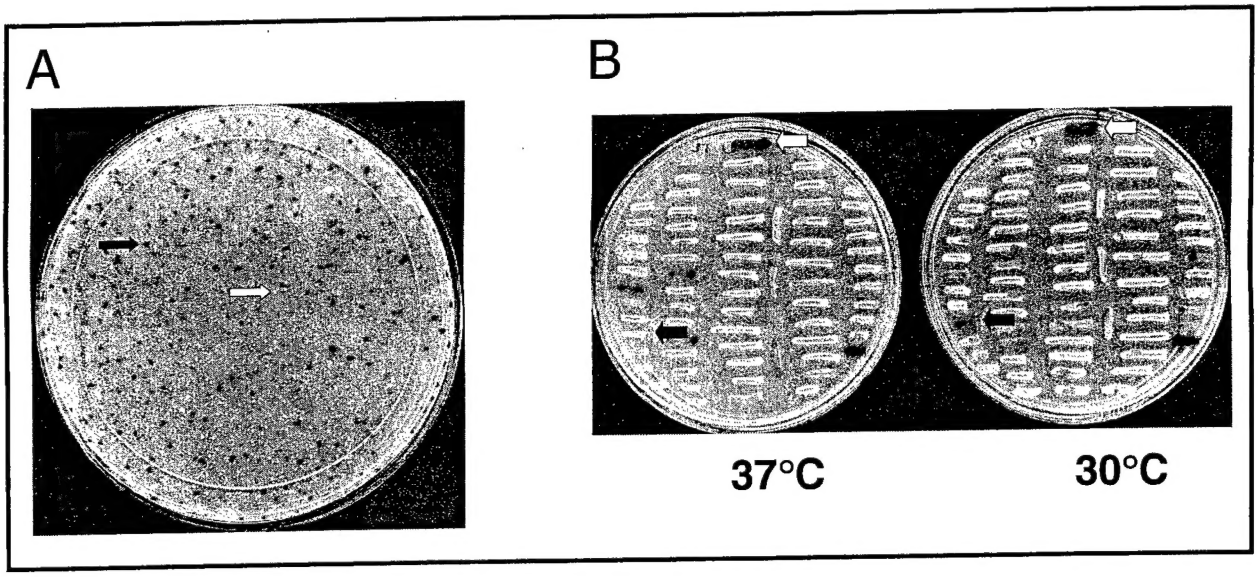


FIGURE 1

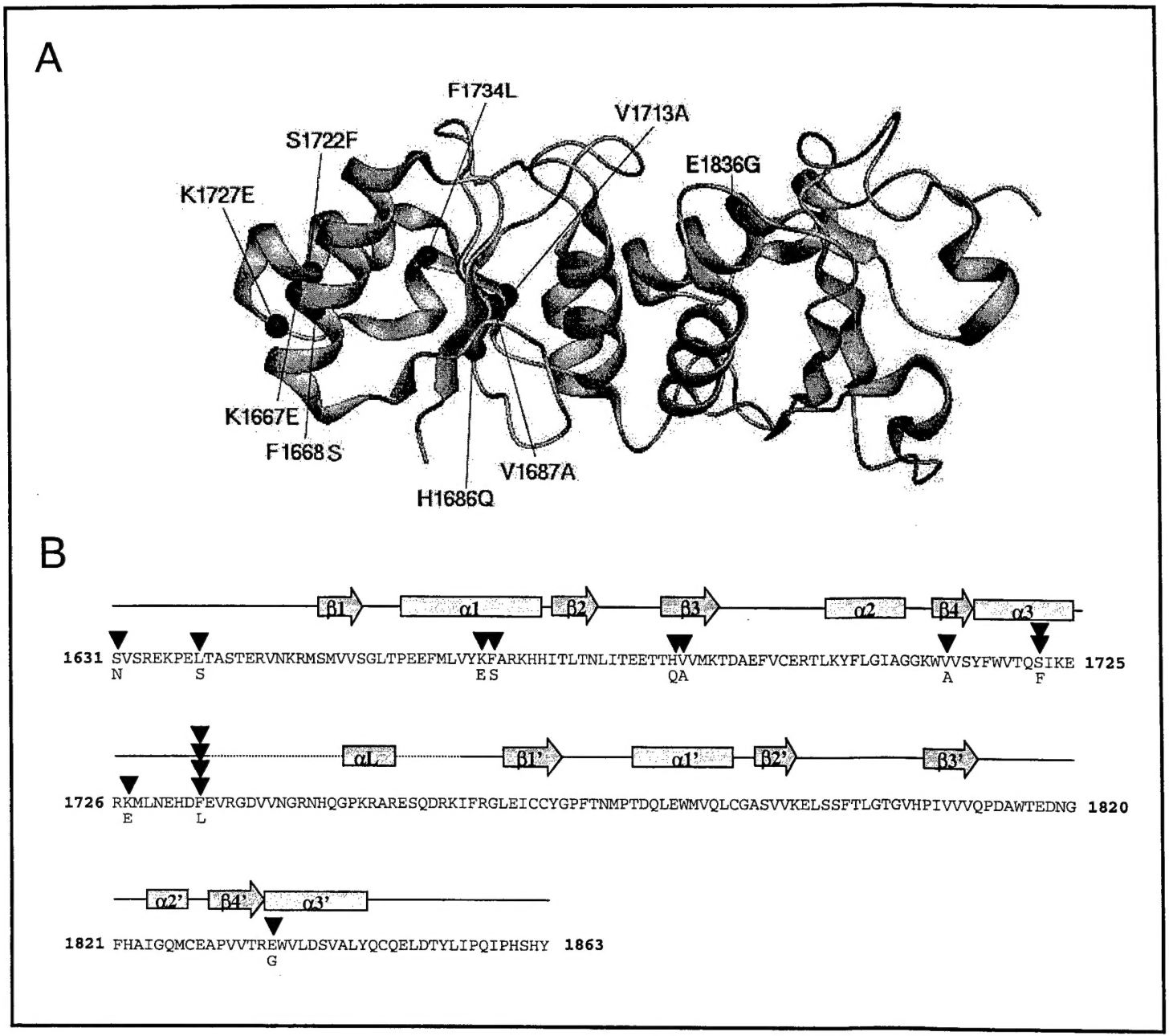

A
F1734L
V1713A
S1722F
E1836G
K1727E
K1667E
F1668S
V1687A
H1686Q
B
β1
α1
β2
β3
α2
β4
α3
1631 SVSREKPELTASTERVNKRMSMVVSGLTPEEFMLVYKFARKHHITLTNLITEETTHVVMKTDAEFVCERTLKYFLGIAGGKWVVSYFWVTQSIKE 1725
N
S
ES
QA
A
F
αL
β1'
α1'
β2'
β3'
1726 RKMLNEHDFEVRGDVVNGRNHQGPKRARESQDRKIFRGLEICCYGPFTNMPTDQLEWMVQLCGASVVKELSSFTLGTGVHPIVVVQPDAWTEDNG 1820
E
L
α2'
β4'
α3'
1821 FHAIGQMCEAPVVTREWVLDSVALYQCQELDTYLIPQIPHSHY 1863
G

FIGURE 2

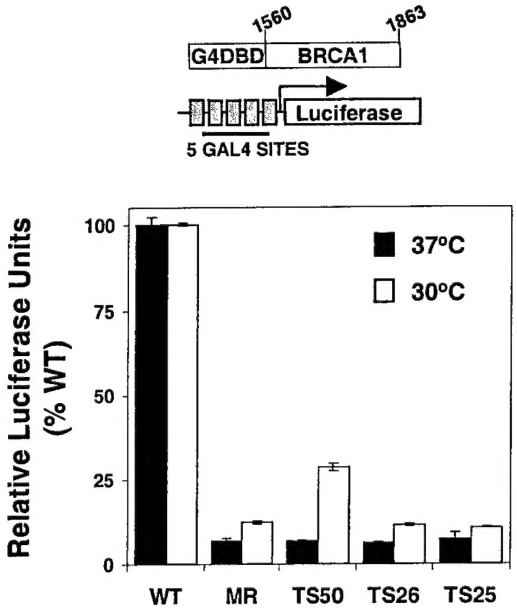

1560
1863
G4DBD
BRCA1
Luciferase
5 GAL4 SITES
Relative Luciferase Units
(% WT)
100
75
50
25
0
37°C
30°C
WT
MR
TS50
TS26
TS25

FIGURE 3

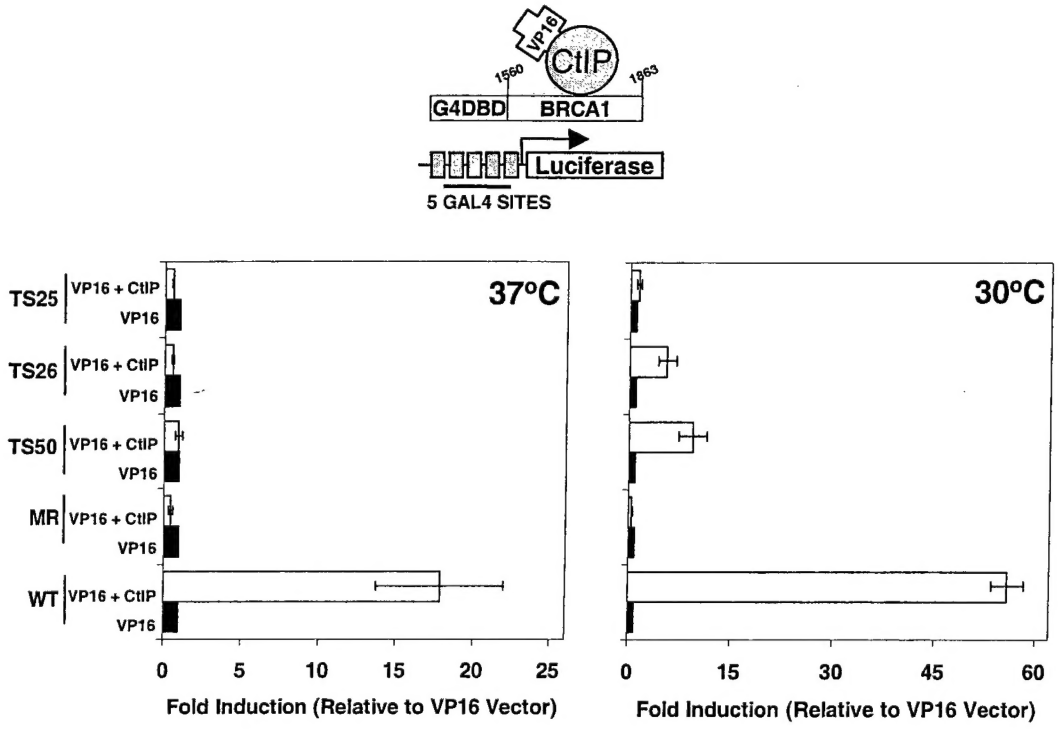

VP16
CtIP
1560
1863
G4DBD
BRCA1
Luciferase
5 GAL4 SITES
TS25
TS26
TS50
MR
WT
VP16 + CtIP
VP16
37°C
30°C
0
5
10
15
20
25
0
15
30
45
60
Fold Induction (Relative to VP16 Vector)
Fold Induction (Relative to VP16 Vector)

FIGURE 4